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Probing Enzyme Stereospecificity. Inhibition of α-Chymotrypsin and Subtilisin Carlsberg by Chiral Amine- and Aminoalcohol-Derivatives

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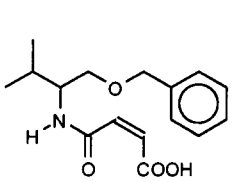
Abstract: Various enantiomeric amine and aminoalcohol amide and α-ketoamide derivatives have been evaluated as competitive inhibitors of the representative serine proteases α-chymotrypsin (CT) and subtilisin Carlsberg (SC). Each compound studied was an effective competitive inhibitor of both enzymes. However, only for the best inhibitor, N-pyruvoyl-1-(1-naphthyl)ethylamine (K_I 27 μM for the *S*-enantiomer with CT), was noteworthy enantiomeric discrimination manifest, with the *S*-enantiomer being a significantly more powerful inhibitor of CT and SC than its *R*-counterpart by factors of 12.6- and 73-fold, respectively. The enzyme-inhibitor interactions responsible for this strong binding and enantiomeric discrimination were revealed by molecular modelling analyses.

INTRODUCTION

Enzymes are now widely used in synthetic organic chemistry, with their abilities to be highly stereoselective in their catalyses being extensively exploited in asymmetric synthesis.[1] However, much remains to be understood regarding the factors responsible for determining the structural and stereospecificity of enzymes towards unnatural substrates. In this regard, we have found studies on competitive inhibitors to provide useful insights into the specificity determinants of enzymes.[2] In this paper, we extend this approach to evaluating the structural and stereospecificity of inhibition of two representative serine proteases, subtilisin Carlsberg (SC, EC 3.4.21.14) and α-chymotrypsin (CT, EC 3.4.21.1) by derivatives of chiral amines. Chiral amines are of general interest in asymmetric synthesis,[3] and occur as structural motifs in many natural products and drugs.[4]

SC and CT are commercially available enzymes that have been extensively applied synthetically[5] and for which high resolution X-ray crystal structures are available.[6,7] In both SC and CT, the

active site binding regions are composed of several subsites, of which the S_1[8]-pocket dominates, particularly in the binding of hydrophobic groups. The interactions of chiral amine derivatives with SC and CT have not been widely studied, although reactions of amino nucleophiles with the acyl enzymes of serine proteases are well documented, [9] and we have previously examined the interactions of some chiral amino alcohols with CT.[10] In this study, the chiral amine derivatives **1-9** are examined as inhibitors of SC and CT.



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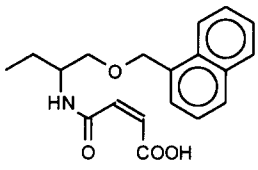

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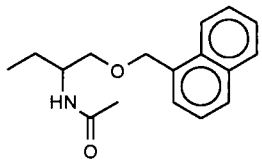

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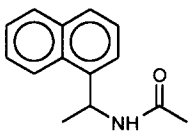

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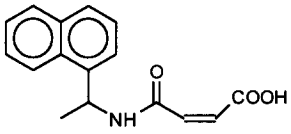

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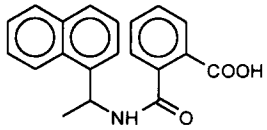

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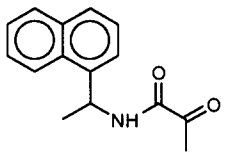

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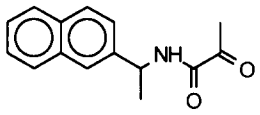

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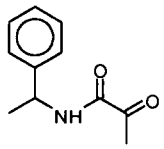


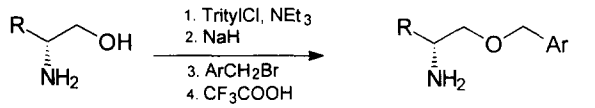
9

Each of these structures possesses an aromatic moiety to provide good binding in the hydrophobic S_1 pockets of the enzymes. The range of amino-acyl functions shown include the acetyl group of **3** and **4** which is widely present in good SC and CT substrates such as N-acetyl phenylalanine methyl ester, the maleyl moiety of **1,2,** and **5**, and the *o*-phthaloyl function of **6**. The latter two groups were employed to confer water solubility on otherwise very hydrophobic structural skeletons. In addition, such N-acyl functions offer the opportunity of additional binding contributions from hydrogen bond formation with active site region amino acid side chains and backbone-peptides. The above structures do not resemble transition state analogs, with the possible

exception of the N-pyruvoyl derivatives **7-9**, for which the keto groups represent good electrophilic centers susceptible to attack by the active site serine, thereby giving rise to tetrahedral transition state analogue enzyme-inhibitor complexes. In fact, α-ketoamide inhibition has been reported for some cysteine proteases,[11] and for aminopeptidases.[12] Furthermore, cyclotheonamide A,[13] a potent natural inhibitor of thrombin, bovine trypsin and human tissue plasminogen activator, contains an α-ketoamide moiety.

RESULTS

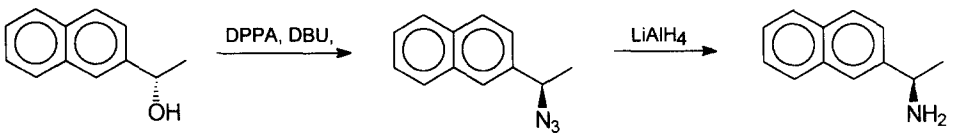
The preparations of the inhibitors were unexceptional. The N-acetyl, N-maleyl, and N-o-phthaloyl derivatives were obtained by treatment of the free amine precursors with the appropriate anhydride. Care had to be taken in purifying and handling all the N-maleyl derivatives because of their highly hygroscopic natures. The enantiomeric amines needed were commercially available, except for the aminoalcohol progenitors of **1R** and **1S**, and **3R** and **3S**. These were prepared from their commercially available enantiomeric amino alcohols precursors, as illustrated for the *R*-series in Scheme 1.



R = $(CH_3)_2CH$ or CH_3CH_2 Ar = phenyl or 1-naphthyl

Scheme 1.

The N-pyruvoyl compounds were obtained from the corresponding amines by EDC-promoted coupling with freshly distilled pyruvic acid. The yields were only in the 25-37% range due to the instability of pyruvic acid. The enantiomeric amine precursors of **7R** and **7S** and **9R** and **9S** were purchased, and the enantiomers of 1-(2-naphthyl)ethylamine prepared as illustrated for the *R*-enantiomer in Scheme 2.[14,15]



Scheme 2

Table 1. Inhibition of α-Chymotrypsin and Subtilisin Carlsberg by **1-9**[a]

Inhibitor	α-Chymotrypsin, K_I (mM)		Subtilisin Carlsberg, K_I (mM)	
	R	*S*	*R*	*S*
1	4.0 ± 1.2	3.2 ± 0.9	2.5 ± 0.2	1.5 ± 0.1
2	1.5 ± 0.4	1.7 ± 0.5	0.98 ± 0.06	0.94 ± 0.05
3	2.4 ± 0.7	1.6 ± 0.5	1.4 ± 0.1	2.2 ± 0.1
4	-[b]	-[b]	-[b]	-[b]
5	6.5 ± 2.0	5.1 ± 1.5	3.3 ± 0.2	4.5 ± 0.3
6	4.4 ± 1.4	7.7 ± 2.2	1.3 ± 0.1	1.3 ± 0.1
7	0.34 ± 0.1	0.027 ± 0.008	2.7 ± 0.2	0.037 ± 0.003
8	0.4 ± 0.1	1.1 ± 0.3	> 10	> 10
9	4.3 ± 1.3	13.7 ± 4.1	18.7 ± 1.3	17.2 ± 1.2

[a] K_I values were determined in duplicate at pH 7.5 in 5% DMSO at 25° C, with Suc-AlaAlaProPhePNA as substrate. [b] Too insoluble to evaluate.

The kinetic data are summarized in Table 1. The parent amines of the Table 1 inhibitors were not evaluated because of the unpredictable effects of amines on enzyme-catalyzed reactions.[16] The N-maleyl, N-acetyl and N-phthaloyl compounds **1-6** are seen to be modest inhibitors of both enzymes, with K_I's similar to those of simple aromatic inhibitors such as 1-naphthol (K_I 0.2 mM).[17] Surprisingly, the K_I's observed within this series vary by less than 7-fold. Furthermore, no significant stereoselectivity is manifest for this group of inhibitors with either enzyme, with the configuration of the stereocenter in any pair of enantiomers exerting very little effect on K_I. Even for the most discriminated pair, **6R** and **6S** with CT, the stereocenter change alters K_I by less than 2-fold. The N-maleyl substituents were adopted in place of the more usual N-acetyl function in order to confer high aqueous solubility on otherwise hydrophobic structures.[18] The necessity for this modification is exemplified by the aqueous insolubility of the N-acetyl derivative **4**, which precluded its evaluation as an inhibitor. In contrast, its N-maleyl counterpart **5** is hygroscopic. In accord with previous experience,[19] the N-acetyl-to-N-maleyl switch does not significantly affect inhibitory power, as exemplified by the similar K_I values for **2,3R** and **2,3S** with both enzymes.

The situation is similar for the ketoamides **8** and **9**, which are at best only modest competitive inhibitors of CT or SC, and for which little enantiomeric discrimination is seen. The maximum effect observed is the 3-

fold difference in K_I's between **9R** and **9S** for CT. In contrast, the enantiomers of the 1-naphthyl ketoamide **7** are good-to-excellent competitive inhibitors of both enzymes. Interestingly, inhibition of CT and SC by the *S*-enantiomer of **7** is significantly superior to that by ***7R***, by factors of 12.6 and 73 respectively. In fact, if the latter degree of enantiomer differentiation were manifest in hydrolysis of a racemic ester-substrate analog of **7**, resolution to give high enantiomeric excesses of residual ester and product acid would be achievable.

Insights into the reasons for the good inhibition properties and enantiomer discrimination of **7** with both enzymes, and particularly the basis for the much greater enantiomer distinction by SC, were sought from molecular modelling. The X-ray structure of the cyclotheonamide A-bovine β-trypsin complex[13] strongly indicates the presence of a covalent, tetrahedral, complex. This is formed by attack of the keto group of the α-ketoamide by the active site serine side chain-OH, with the negative charge of the O^- generated being stabilized by the oxyanion hole. This information was used to guide the molecular modelling strategy. Firstly, the X-ray structures of SC and CT were energy-minimized by molecular mechanics and molecular dynamics. Each of ***7R*** and ***7S*** was then docked into each active site, with the naphthyl moieties in S_1, and the α-keto carbonyl carbon covalently connected to the active site serine-CH_2OH oxygen to form a transition state-like tetrahedral intermediate. Each EI-complex was then subjected to energy-minimization by molecular mechanics, followed by molecular dynamics, calculations and the optimized conformations of each *R*- and *S*-pair of EI-complexes compared. The results are depicted in Figures 1 and 2. In each minimized EI-complex, there were strong interactions between the oxyanion of the tetrahedral intermediate and the oxyanion hole H-bonding residues of the peptide backbone NH's of Ser195 and Gly193 of CT, and the backbone NH of Ser221 and the side chain NH_2 of Asn155 of SC, respectively.

For CT (Figure 1), the naphthyl residues of both ***7R*** and ***7S*** penetrate virtually equivalently into the S_1-pocket to provide good hydrophobic binding contributions. The 13-fold binding advantage of the *S*-enantiomer is seen to be due to the fact that its methyl group is oriented towards the indole residue of Trp215, thereby eliciting a hydrophobic binding contribution that is absent in the EI-complex of the ***7R***-enantiomer, for which the methyl substituent is directed towards the external solvent.

For binding of ***7R*** and ***7S*** to SC, the favoring of the *S*-enantiomer is even more clear cut (Figure 2). Again, the methyl group at the stereocenter of ***7S*** elicits favorable hydrophobic interactions by its orientation towards the oily Leu126 side chain. In contrast, the corresponding methyl group of ***7R*** is oriented towards the external water in a manner that does not contribute to binding. In addition, the naphthyl group of the *S*-enantiomer is now able to locate itself deeper inside the S_1-pocket than can its enantiomeric equivalent,

thereby providing a stronger naphthyl-S_1 contribution to binding. It is the synergistic combination of these two factors that accounts for the greater (73-fold) difference in K_I's between ***7S*** and ***7R*** as inhibitors of SC.

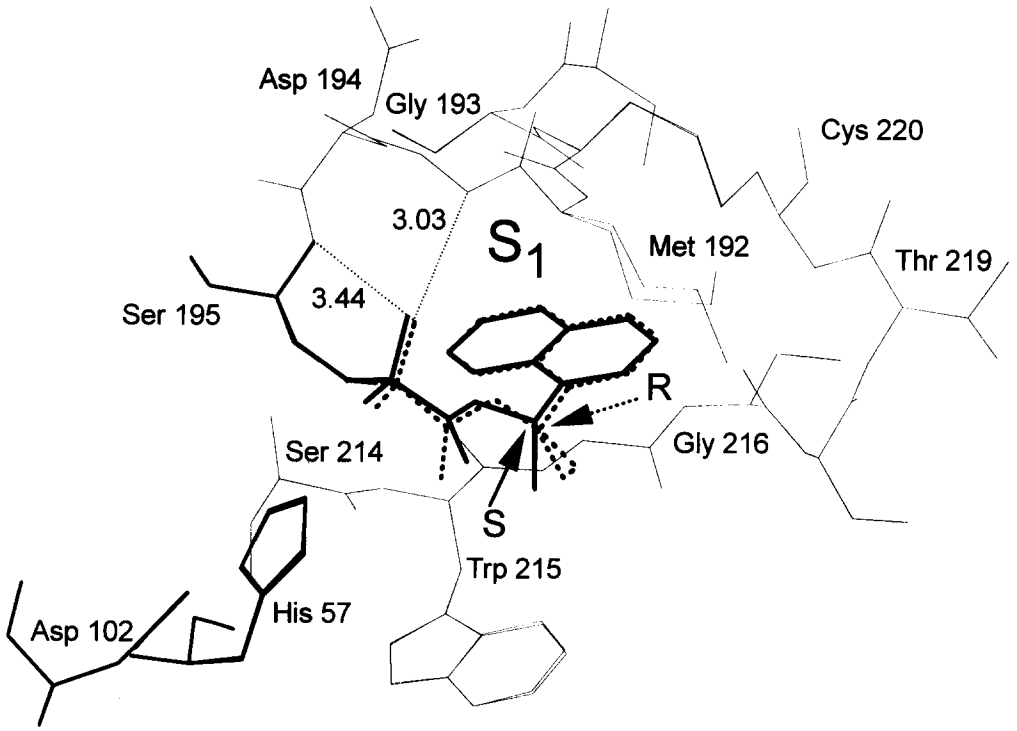


Figure 1. Superimposed energy-minimized, EI-complexes of *S*- and *R*-N-pyruvoyl-1-(1-naphthyl)ethylamine, ***7S*** (▬▬)and ***7R*** (●●●●) respectively, in the active site of CT. The oxyanions of the tetrahedral complexes derived from both ***7S*** and ***7R*** are located in the oxyanion hole, with the negative charges stabilized by hydrogen-bonding (·····) with the peptide NH's of Ser195 (O^--N, 3.44 Å) and Gly193 (O^--N, 3.03Å). Both naphthyl groups are positioned in the hydrophobic S_1 pocket. The main difference between the two orientations is that the methyl group at the stereocenter of ***7S*** (▬▶) is located close to the Trp215 side chain, thereby making a favorable hydrophobic binding contribution, while that of ***7R*** (·····▶) is oriented towards the outside of the active site and does not contribute to binding.

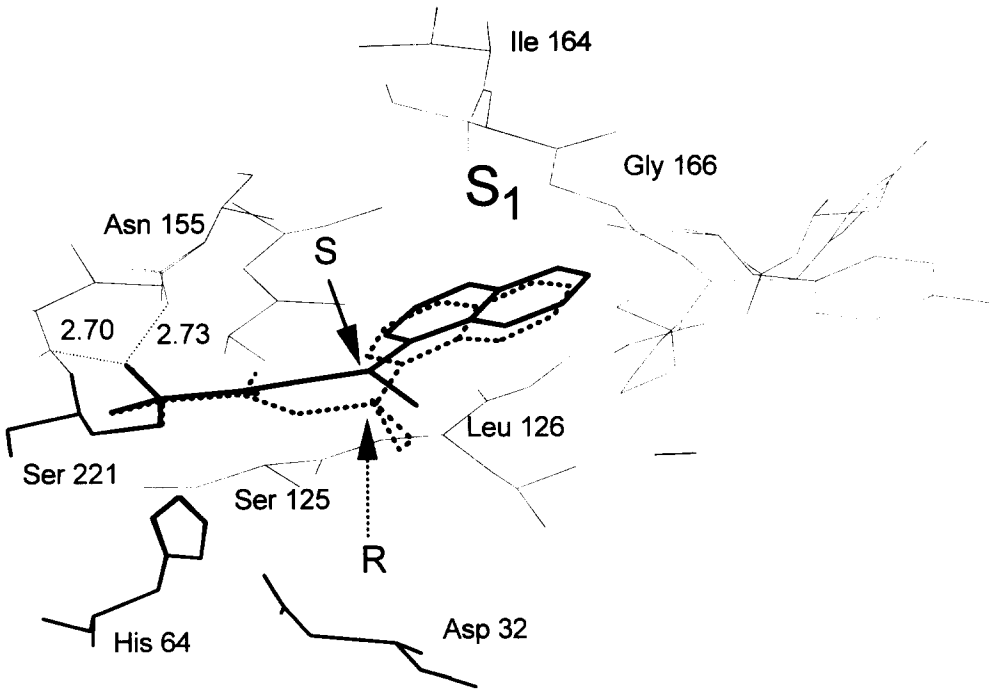


Figure 2. Superimposed energy-minimized, EI-complexes of *S*- and *R*-N-pyruvoyl-1-(1-naphthyl)ethylamine, ***7S*** (▬▬)and ***7R*** (●●●●) respectively, in the active site of SC. The oxyanions of the tetrahedral complexes derived from both ***7S*** and ***7R*** are located in the oxyanion hole, with the negative charges stabilized by hydrogen-bonding (·····) with the peptide NH of Ser221 (O^--N, 2.70 Å) and the side chain NH_2 of Asn155 (O^--N, 2.73 Å). While both naphthyl groups are positioned in the hydrophobic S_1 pocket, that of the *S*-enantiomer is more deeply, and more favorably, located. Another difference between the two orientations is that the orientation of the methyl group at the stereocenter of ***7S*** (▬▶) towards the Leu126 side chain makes a favorable hydrophobic binding contribution, while that of ***7R*** (·····▶) does not contribute to binding because it is oriented towards the external solvent.

Intriguingly, when the naphthyl group of **7** is replaced by a phenyl residue, as in **9**, enantiomeric discrimination by SC and CT is dramatically reduced, being non-existent for SC and only 3-fold for CT. Furthermore, the direction of enantiomer preference is reversed relative to **7**. Molecular modelling was not applied to try to explain the basis for the reduced enantiomer recognition and stereoselectivity reversal for ***9R*** and ***9S*** because the energy differences involved would be too small for dependable conclusions to be drawn.

While the α-ketoamide function has proven to be a promising functional group for eliciting competitive inhibition responses, the markedly different inhibition properties of the enantiomers of the 1-naphthyl (**7**) and 2-

naphthyl (**8**) compounds demonstrate that the structural specificity criteria for this series are sensitive and strict. Further delineation of this aspect is planned, as are experiments to ascertain if the types of enantiomer binding differences noted in Figure 2 can be translated into SC-catalyzed resolutions of racemic α-ketoester substrate analogs of **7**.

EXPERIMENTAL

Flash chromatography was performed using Si gel 60 (40-63 μ), supplied by Toronto Research Chemicals Inc. M.p's were obtained using a Fisher-Johns apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer 882 Infrared Spectrophotometer and NMR spectra on a Varian Gemini 200 spectrometer. Chemical shifts are reported in ppm relative to the TMS peak (δ = 0.00). MS spectra were obtained with Bell and Howell 21-490 (low resolution) and AEI MS3074 (high resolution) instruments. Optical rotations were determined on a Perkin Elmer 243B polarimeter. (1*R*)- and (1*S*)-1-(1-Naphthyl)ethylamine, (2*R*)- and (2*S*)-2-aminobutanol, (2*R*)- and (2*S*)-2-amino-3-methylbutanol, (1*R*)- and (1*S*)-1-(2-naphthyl)ethanol, and N-SuccAlaAlaProPhe-p-nitroanilide were purchased from Aldrich. α-Chymotrypsin was purchased from ICN Pharmaceutical (lot 6373), and subtilisin Carlsberg from Sigma (lot 29F-0050).

Preparations of Amide Inhibitors 1-6.

***R*-Series**

(2*R*)-N-Maleyl-O-benzyl-2-amino-3-methylbutanol (1*R*).

A solution of triphenylchloromethane (593 mg, 2.13 mmoles) in $CHCl_3$ (2 mL) was slowly added to a mixture of (2*R*)-2-amino-3-methylbutanol (220 mg, 2.13 mmoles) and NEt_3 (215 mg, 2.13 mmoles) in $CHCl_3$ (2 mL), with stirring at 0°C and under N_2. The stirring was continued for 24 h at 0 °C. The mixture was then washed with 10 % aqueous citric acid (2 x 5 mL), followed by water (5 mL), and dried (Na_2SO_4). After filtration and removal of the solvent, the trityl derivative was obtained as a pale brown gummy solid (725 mg): ^1H NMR ($CDCl_3$) δ: 7.60-7.50 (m, 6 H), 7.35-7.10 (m, 9 H), 3.34 (J_{AB} = 11.0 Hz, J_{AX} = 3.4 Hz, 1 H), 3.11 (J_{AB} = 11.0 Hz, J_{BX} = 5.0 Hz, 1 H), 2.43 (m, 1 H), 2.07 (br s, 1 H), 1.25 (m, 1 H), 0.81 (d, J = 7.0 Hz, 3 H), 0.66 (d, J = 6.9 Hz, 3 H). This material was then dissolved in anhydrous DMF (10 mL) at 0 °C under N_2 and treated with NaH (170.4 mg, 4.26 mmoles). The mixture was allowed to warm slowly to 20 °C during 1.5 h and *n*-Bu_4NI (7.86 mg, 0.021 mmoles) and benzyl bromide (402 mg, 2.35 mmoles) then added, with stirring. After further stirring for 8 h, the mixture was cooled to 0 °C, and 30 mL of water were added cautiously. The mixture

was extracted with Et_2O (2 x 25 mL), the organic phase washed with water and then dried (Na_2SO_4). Rotoevaporation afforded the trityl benzyl ether derivative as a brown oil (1.12 g): ^1H NMR ($CDCl_3$) δ: 7.70-7.10 (m, 20 H), 4.57 (s, 2 H), 4.09 (m, 2 H), 3.07 (m, 1 H), 1.82 (m, 1 H), 1.54 (s, 1 H), 0.91 (d, J = 7.0 Hz, 3 H), 0.78 (d, J = 7.0 Hz, 3 H).

This product was dissolved in $CHCl_3$ (3 mL) and anhydrous MeOH (8 mL) at -5°C under N_2, and CF_3COOH (8 mL) added, with stirring for 3 h, after which the mixture was allowed to warm to 20 °C. After 12 h, the solvent was rotoevaporated, water (30 mL) added to the residue, and the mixture extracted with Et_2O (3 x 20 mL). The combined organic layers were washed with 5% aqueous HCl (2 x 20 mL). All the aqueous layers were combined, cooled to 5 °C, and made alkaline by adding a few NaOH pellets. This aqueous solution was saturated with NaCl, extracted with Et_2O (5 x 15 mL), the combined organic layers dried (Na_2SO_4). After filtration, the solvent was removed by distillation at 1 atm, and the residue Kugelrohr-distilled (0.25 mmHg, 70-90 °C), to give (2*R*)-O-benzyl-2-amino-3-methylbutanol as colorless liquid (178 mg): $[\alpha]^{25}_D$ -15.3° (c = 0.58, $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 7.38-7.20 (m, 5 H), 4.53 (s, 2 H), 3.53 (J_{AB} = 9.0 Hz, J_{AX} = 4.0 Hz, 1 H), 3.30 (J_{AB} = 9.0 Hz, J_{BX} = 8.8 Hz, 1 H), 2.78 (m, 1 H), 1.65 (m, 1 H), 1.40 (br s,2 H), 0.91 (d, J = 7.0 Hz, 6 H); IR 3378 cm^{-1}. MS *m/z* (rel int) 150 (36), 112 (30), 92 (18), 91 (100), 73 (10), 72 (95), 65 (17), 55 (28). HRMS: calcd for $C_{12}H_{19}NO{\cdot}H^+$ (MH^{+}) 194.15449. Found 194.15474.

Maleic anhydride (374 mg, 3.82 mmoles) was added all at once with stirring to (2*R*)-O-benzyl-2-amino-3-methylbutanol (737 mg, 3.82 mmoles) in anhydrous Et_2O (20 mL) at 0 °C under N_2. After stirring for 2 h at 0 °C, the mixture was allowed to warm to 20 °C and kept for 12 h, when the solvent was rotoevaporated, and the oily residue dissolved in saturated aqueous $NaHCO_3$ (20 mL) and washed with Et_2O (2 x 20 mL). The aqueous phase was acidified to pH 2 with 10% aqueous HCl, extracted with Et_2O (3 x 20 mL), the organic layer dried (Na_2SO_4), then filtered and evaporated to give (2*R*)-N-maleyl-O-benzyl-2-amino-3-methylbutanol (**1*R***) as a white solid (892 mg): mp 64-65 °C; $[\alpha]^{25}_D$ +75.0° (c = 0.86, MeOH); ^1H NMR (d_6-DMSO) δ: 9.20 (br d, J = 9.4 Hz, 1 H), 7.40-7.20 (m, 5 H), 6.45 (J_{AB} = 12.6 Hz, 1 H), 6.26 (J_{AB} = 12.6 Hz, 1 H), 4.51 (J_{AB} = 11.9 Hz, 1 H), 4.45 (J_{AB} = 11.9 Hz, 1 H), 3.83 (m, 1H), 3.48 (d, J = 5.4 Hz, 2 H), 1.90 (m, 1 H), 0.86 (m, 6 H); IR 3292-2870, 1706, 1630 cm^{-1}. Anal. calcd for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.26. Found : C, 65.61, H, 7.33.

The following *R*-inhibitors were prepared analogously, as follows:

(2*R*)-N-Maleyl-O-(1-naphthylmethyl)-2-aminobutanol (2*R*).

From triphenylchloromethane (1.74 g, 6.2 mmoles) and (2*R*)-2-aminobutanol (552 mg, 6.2 mmoles) to give the trityl derivative (2.05 g) as a pale brown gummy solid; ^{1}H NMR ($CDCl_3$) δ: 7.60-7.10 (m, 15 H), 3.24 (J_{AB} = 10.8 Hz, J_{AX} = 3.2 Hz, 1 H), 3.04 (J_{AB} = 10.8 Hz, J_{BX} = 4.2 Hz, 1 H), 2.50 (m, 1 H), 1.97 (br s, 1 H), 1.03-0.80 (m, 2 H), 0.62 (t, J = 7.3 Hz, 3 H).

From this material and 1-bromomethylnaphthalene (1.39 g, 6.3 mmoles) was obtained the corresponding trityl naphthylmethyl ether as an oil (3.02 g); ^{1}H NMR ($CDCl_3$) δ: 8.04 (m, 1H), 7.90-7.70 (m, 2 H), 7.60-7.05 (m, 19 H), 4.66 (s, 2 H), 3.07 (m, 2 H), 2.55 (m, 1 H), 1.40-1.28 (m, 2 H), 0.64 (t, J = 7.3 Hz, 3 H).

Deprotection of this material gave (2*R*)-O-(1-naphthylmethyl)-2-aminobutanol (886 mg), Kugelrohr b.p. 95-110 °C (0.05 mmHg), $[\alpha]^{25}_D$ -11.9° (c = 0.77, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ: 8.10 (m,1 H), 7.90-7.75 (m, 2 H), 7.58-7.35 (m, 4 H), 4.97 (s, 2 H), 3.55 (J_{AB} = 9.0 Hz, J_{AX} = 3.8 Hz, 1 H), 3.32 (J_{AB} = 9.0 Hz, J_{BX} = 7.8 Hz, 1 H), 2.90 (m, 1 H), 1.55-1.19 (m, 2 H), 1.39 (s, 2 H), 0.91 (t, J = 7.4 Hz, 3 H); IR 3374 cm^{-1}; HRMS: calcd for $C_{15}H_{19}NO$ 229.14666. Found 229.14627.

The above amine (500 mg, 2.18 mmoles) and maleic anhydride (213 mg, 2.18 mmoles) yielded (2*R*)-N-maleyl-O-(1-naphthylmethyl)-2-aminobutanol (**2*R***) as a white solid (523 mg): mp 98-99 °C; $[\alpha]^{25}_D$ +96.9° (c = 0.55, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ: 8.12 (m, 1H), 7.92-7.80 (m, 2 H), 7.60-7.38 (m, 4 H), 6.20 (br s, 1 H), 6.19 (d, J = 12.8 Hz, 1 H), 5.58 (d, J = 12.8 Hz, 1 H), 5.07 (J_{AB} = 12.0 Hz, 1 H), 4.88 (J_{AB} = 12.0 Hz, 1 H), 3.95 (m, 1 H), 3.64 (J_{AB} = 9.7 Hz, J_{AX} = 3.0 Hz, 1 H), 3.54 (J_{AB} = 9.7 Hz, J_{BX} = 3.4 Hz, 1 H), 1.60 (m, 2 H), 0.87 (t, J = 7.4 Hz, 3 H); IR 3416-2878, 1713 cm^{-1}. Anal. calcd for $C_{19}H_{21}NO_4$: C, 69.70; H, 6.47. Found: C, 69.56; H, 6.70.

(2*R*)-N-Acetyl-O-(1-naphthylmethyl)-2-aminobutanol (3*R*).

(2*R*)-O-(1-naphthylmethyl)-2-aminobutanol (229 mg, 1 mmol) and Ac_2O (115 mg, 1.1 mmoles) gave **3*R*** (200 mg): mp 70-71 °C; $[\alpha]^{25}_D$ -63.5° (c = 0.55, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ: 8.12 (m, 1 H), 7.92-7.78 (m, 2 H), 7.60-7.38 (m, 4 H), 5.52 (d, J = 8.1 Hz, 1 H), 4.98 (J_{AB} = 11.9 Hz, 1 H), 4.91 (J_{AB} = 11.9 Hz, 1 H), 3.95 (m, 1 H), 3.57 (J_{AB} = 9.4 Hz, J_{AX} = 3.1 Hz, 1 H), 3.51 (J_{AB} = 9.4 Hz, J_{BX} = 3.7 Hz, 1 H), 1.79 (s, 3 H), 1.53 (m, 2 H), 0.85 (t, J = 7.3 Hz, 3 H); IR 3438, 1659, 1596, cm^{-1}. Anal. calcd for $C_{17}H_{21}NO_2$: C, 75.25; H, 7.80. Found: C, 75.13; H, 7.88.

(1*R*)-N-Acetyl-1-(1-naphthyl)ethylamine (4*R*).

1*R*-(1-Naphthyl)ethylamine (410 mg, 2.4 mmoles) and Ac_2O (264 mg, 2.6 mmoles) afforded **4*R***, white needles from CCl_4 (464 mg, 91%): mp 153-154 °C; $[\alpha]^{25}_D$ + 122.9° (c = 0.62, $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 8.09 (m, 1 H), 7.90-7.75 (m, 2 H), 7.60-7.39 (m, 4 H), 5.89 (m, 1 H), 5.63 (br s, 1 H), 1.95 (s, 3 H), 1.66 (d, *J* = 6.7 Hz, 3 H); IR 3437, 3007, 1658, 1497 cm^{-1}. HRMS: calcd for $C_{14}H_{15}NO$ 213.11536. Found 213.11536.

(1*R*)-N-Maleyl-1-(1-naphthyl)ethylamine (5*R*).

Maleic anhydride (235 mg, 2.4 mmoles) and (1*R*)-1-(1-naphthyl)ethylamine (410 mg, 2.4 mmoles) gave **5*R*** (570 mg, after recrystallization from Et_2O/MeOH) : mp 139-140 °C: $[\alpha]^{25}_D$ -64.1° (c = 0.98, MeOH); ^1H NMR ($CDCl_3$) δ 8.01 (d, *J* = 8.0 Hz, 1 H), 7.86 (m, 2 H), 7.55 (m, 4 H), 7.16 (br d, *J* = 8.2 Hz, 1 H), 6.24 (J_{AB} = 14.3 Hz, 1 H), 6.21 (J_{AB} = 14.3 Hz, 1 H), 5.97 (m, 1 H),1.76 (d, *J* = 6.8 Hz, 3 H); IR 3419-2000, 1711, 1631, 1582 cm^{-1}. Anal. calcd for $C_{16}H_{15}NO_3$: C, 71.36; H, 5.61. Found: C, 71.62; H, 5.62.

(1*R*)-N-Phthaloyl-1-(1-naphthyl)ethylamine (6*R*).

Phthalic anhydride (86.5 mg, 0.59 mmoles) and (1*R*)-1-(1-naphthyl)ethylamine (100 mg, 0.59 mmoles) in anhydrous THF (5 mL) yielded a white solid that was purified by dissolving in saturated aqueous $NaHCO_3$ (20 mL), extracting with ethyl acetate (2 x 20 mL), then acidifying to pH 2.5 with 6M aqueous HCl. The resulting white precipitate was filtered, washed with water, and then dried in vacuo under P_2O_5 to give **6*R*** (127 mg): mp 160-161 °C; $[\alpha]^{25}_D$ -45.1° (c = 0.46, MeOH); ^1H NMR ($CDCl_3$) δ: 8.22 (d, *J* = 8.4 Hz, 1 H), 8.15 (m, 1 H), 7.87 (m, 2 H), 7.70-7.35 (m, 7 H), 6.34 (br d, *J* = 7.7 Hz, 1 H), 6.15 (m, 1 H), 1.83 (d, *J* = 6.6 Hz, 3 H); IR 3428 (br), 1724, 1700, 1662, 1583 cm^{-1}. Anal. calcd for $2C_{20}H_{17}NO_3 \cdot 1H_20$: C, 73.16; H, 5.52. Found: C, 73.33; H, 5.21.

S-Series

The *S*-amide inhibitors were prepared, spectroscopically identical and in the same general yields, as for the corresponding *R*-inhibitors above, and had the following properties:

(2*S*)-N-Maleyl-O-benzyl-2-amino-3-methylbutanol (1*S*): mp 64-65 °C; $[\alpha]^{25}_D$ -75.1° (c = 0.83, MeOH). Anal. calcd for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.26. Found :C, 65.78; H, 7.24.

(*S*)-N-Maleyl-O-(1-naphthylmethyl)-2-aminobutanol (2*S*): mp 98-99 °C; $[\alpha]^{25}_D$ -96.7° (c = 0.67, $CHCl_3$). Anal. calcd for $C_{19}H_{21}NO_4$: C, 69.70; H, 6.47. Found: C, 69.48; H, 6.77.

(2*S*)-N-Acetyl-O-(1-naphthylmethyl)-2-aminobutanol (3*S*): mp 70-71 °C; $[\alpha]^{25}_D$ +63.4° (c = 0.61, $CHCl_3$). Anal. calcd for $C_{17}H_{21}NO_2$: C, 75.25; H, 7.80. Found: C, 75.07; H, 7.96.

(1*S*)-(-)-N-Acetyl-1-(1-naphthyl)ethylamine (4*S*): mp 153-154 °C; $[\alpha]^{25}_D$ -125.0° (c = 1.16, $CHCl_3$). HRMS: calcd for $C_{14}H_{15}NO$ 213.11536. Found 213.11562.

(1*S*)-N-Maleyl-1-(1-naphthyl)ethylamine (5*S*): mp 139-140 °C; $[\alpha]^{25}_D$ +64.0° (c = 0.98, MeOH). Anal. calcd for $C_{16}H_{15}NO_3$: C, 71.36; H, 5.61. Found: C, 71.58; H, 5.55.

(1*S*)-N-Phthaloyl-1-(1-naphthyl)ethylamine (6*S*): mp 160-161 °C; $[\alpha]^{25}_D$ +45.2° (c = 0.49, MeOH). Anal. calcd for $2C_{20}H_{17}NO_3.1H_20$: C, 73.16; H, 5.52. Found: C, 73.14; H, 5.25.

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***R*-Series**

(1*R*)-N-Pyruvoyl-1-(1-naphthyl)ethylamine (7*R*).

EDC (422 mg, 2.2 mmoles) was added at 0 °C with stirring under N_2 to (1*R*)-1-(1-naphthyl)ethylamine (342 mg, 2 mmoles) and freshly distilled $CH_3COCOOH$ (193 mg, 2.2 mmoles) in dry CH_2Cl_2. After keeping for 30 min at 0 °C, the mixture was allowed to warm to 20 °C and stirred for 18 h. CH_2Cl_2 (50 mL) was then added, the mixture washed with 10 % aqueous HCl (3 x 50 mL), then with saturated aqueous $NaHCO_3$ (2 x 50 mL) and finally with brine (50 mL). The organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo, and the gummy residue flash-chromatographed (CH_2Cl_2 elution), then Kugelrohr-distilled (135-145 °C, 0.025 mmHg) to give **7*R*** (146 mg): $[\alpha]^{25}_D$ +48.0° (c = 0.57, $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 8.10-8.01 (m, 2 H), 7.95-7.78 (m, 2 H), 7.60-7.40 (m, 4 H), 7.20 (br s, 1 H), 5.88 (m, 1 H), 2.49 (s, 3 H), 1.70 (d, *J* = 6.9 Hz, 3 H); IR 3380, 1710, 1680, 1510 cm^{-1}. Anal. calcd for $C_{15}H_{15}NO_2$: C, 74.67; H, 6.27. Found: C, 74.48; H, 6.40.

(1*R*)-N-Pyruvoyl-1-phenylethylamine (9*R*) was prepared analogously from freshly distilled $CH_3COCOOH$ (354 mg, 4 mmoles) and (1*R*)-1-phenylethylamine (242.4 mg, 2 mmoles) to give, after chromatography followed by recrystallization from hexane, **9*R*** (158 mg): mp 70-71 °C; $[\alpha]^{25}_D$ +106.8° (c = 0.33, $CHCl_3$); ^1H

NMR ($CDCl_3$) δ: 7.40-7.25 (m, 5 H), 7.15 (br s, 1 H), 5.05 (m, 1 H), 2.47 (s, 3 H), 1.54 (d, J = 7.1 Hz, 3 H); IR 3370, 1705, 1683 cm^{-1}. Anal. calcd. for $C_{11}H_{13}NO$: C, 69.09; H: 6.85. Found: C, 68.99; H, 6.91.

(1*R*)-N-Pyruvoyl-1-(2-naphthyl)ethylamine (8*R*).

DBU (1.04 mL, 6.97 mmoles) was added, at 5 °C with stirring under N_2, to (1*S*)-1-(2-naphthyl)ethanol (1g, 5.81 mmoles) and diphenylphosphoryl azide (1.92 g, 6.97 mmoles) in dry toluene (10 mL). The mixture was stirred for a further 2 h at 5 °C, and then for 16 h at 20 °C. The resulting two phase mixture was washed with water (2 x 10 mL) and then with 5% aqueous HCl (10 mL). The organic layer was rotoevaporated to give the corresponding (1*R*)-azide (1.565 g, used directly in the next step): ^1H NMR ($CDCl_3$) δ: 7.90-7.75 (m, 4 H), 7.55-7.40 (m, 3 H), 4.79 (q, J = 6.6 Hz, 1 H), 1.62 (d, J = 6.6 Hz, 3 H).

The above azide (1.55 g, 7.8 mmoles) in dry Et_2O (60 mL) was added with stirring under N_2 to $LiAlH_4$ (440 mg, 11.6 mmoles) in dry Et_2O (150 mL) such that reflux was maintained. The mixture was refluxed for a further 2 h and then kept at 20 °C for 12 h. Moist ether was added to destroy the excess of $LiAlH_4$, then water (5 mL) added, followed by 10% aqueous NaOH (10 mL). The mixture was filtered and the organic layer dried (Na_2SO_4). The solvent was rotoevaporated, the resulting solid dissolved in 5% aqueous HCl, the solution washed with Et_2O (2 x 30 mL), the aqueous phase brought to pH 9 with 10% aqueous NaOH, and extracted with Et_2O (3 x 30 mL). The latter organic layer was dried (Na_2SO_4), filtered and evaporated, to give (1*R*)-1-(2-naphthyl)ethylamine as a white solid (961 mg): mp 52-53 °C; $[\alpha]^{25}_D$ +18.5 (c = 0.54, EtOH) (lit.[20] m.p. 52-53 °C, $[\alpha]^{25}_D$ +19 (EtOH)) ; ^1H NMR ($CDCl_3$) δ: 7.90-7.75 (m, 4 H), 7.55-7.40 (m, 3 H), 4.30 (q, J = 6.6 Hz, 1 H), 1.75 (br s, 2 H), 1.48 (d, J = 6.6 Hz, 3 H); IR 3400 cm^{-1}.

Pyruvic acid (354 mg, 4 mmoles) was added dropwise with stirring at 0 °C under N_2 to the above (1*R*)-amine (343 mg, 2 mmoles) and EDC (383 mg, 2 mmoles) in dry CH_2Cl_2 (18 mL). After stirring for 30 min at 0 °C, the mixture was allowed to warm to 20 °C and stirred overnight. Work-up and purification as for **7*R*** above afforded **8R** (163 mg after recrystallization from hexane): mp 105-106 °C; $[\alpha]^{25}_D$ +140.1 ° (c = 0.43, $CHCl_3$); ^1H NMR ($CDCl_3$) δ: 7.90-7.75 (m, 4 H), 7.50-7.35 (m, 3 H), 7.26 (br d, 1 H), 5.22 (m, 1 H), 2.48 (s, 3 H), 1.63 (d, J = 6.9 Hz, 3 H); IR 3380, 1710, 1680 cm^{-1}. Anal. calcd for $C_{15}H_{15}NO_2$: C, 74.67; H, 6.27. Found: C, 74.43; H, 6.47.

S-Series

The *S*-amide inhibitors were prepared, spectroscopically identical and in the same general yields, as for the corresponding *R*-inhibitors above, and had the following properties:

(1*S*)- N-Pyruvoyl-1-(1-naphthyl)ethylamine (7*S*): Kugelrohr b.p. 135-145 °C (0.025 mmHg); $[\alpha]^{25}_{D}$ -47.5 ° (c = 0.54, $CHCl_3$). Anal. calcd for $C_{15}H_{15}NO_2$: C, 74.67; H, 6.27. Found: C, 74.45; H, 6.39.

(1*S*)-(-)-N-Pyruvoyl-1-(2-naphthyl)ethylamine (8*S*): *via* (1*S*)-1-(2-naphthyl)ethylamine; mp 52-53 °C; $[\alpha]^{25}_{D}$ -18.6° (c = 0.56, EtOH) (lit.[20] m.p. 52-53 °C, $[\alpha]^{25}_{D}$ -19 (EtOH)), to give **8*S***: mp 105-106 °C; $[\alpha]^{25}_{D}$ -142.8° (c = 0.69, $CHCl_3$). Anal. calcd for $C_{15}H_{15}NO_2$: C, 74.67; H, 6.27. Found: C, 74.40; H, 6.47.

(1*S*)-(-)-N-Pyruvoyl-1-phenylethylamine (9*S*): mp 70-71°C; $[\alpha]^{25}_{D}$ -98.6° (c = 0.37, $CHCl_3$). Anal. calcd. for $C_{11}H_{13}NO$: C, 69.09; H: 6.85. Found: C, 68.92; H, 7.00.

Kinetic Studies

The kinetic measurements, and the K_I determinations by the method of Waley,[21] were performed as detailed previously[22] at 25 °C in 0.1 M phosphate buffer of pH 7.5 containing 0.5 M NaCl and 5% DMSO, and using N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as the reference substrate. Enzyme concentrations were 2 x 10^{-8} M for CT and 4.5 x 10^{-9} M for SC. The substrate concentration range was 0.25-10 x 10^{-4} M for CT and 0.30-20 x 10^{-4} M for SC. Inhibitor concentrations ranged from 1x10^{-2} to 1x10^{-4} M. All runs were done in duplicate, and the data were analyzed using the Grafit program (Erithacus Software Ltd., UK). The results are recorded in Table 1.

Molecular Modelling.

The reference structures used for SC and CT were from the Protein Data Bank[23] at Brookhaven National Laboratory,[24] with Insight II (version 2.3.0, Biosym Technologies, Inc., San Diego, CA, USA) as the graphics software on Silicon Graphics 240 GTX and Indigo computers. The system was set up, and minimizations of each enzyme performed, with the DISCOVER program (Biosym, Version 3.1) using the Consistent Valence Force Field (CVFF),[25] as described in detail previously.[22]

The EI-complex minimizations of CT and SC with **7*R*** and **7*S*** were performed by the previous protocols,[22] after docking each ketoamide inhibitor, and creating a chemical bond between the Oγ of each active site serine and the ketone carbon of the pyruvoyl moiety, again as described earlier for aldehyde inhibitors.[22] The energy-minimized structures obtained are depicted in Figures 1 and 2.

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REFERENCES

1. (a) *Introduction to Using Biocatalysis Using Enzymes and Microorganisms,* Roberts, S.M.; Turner, N.J.; Willetts, A.J.; Turner, M.K., Cambridge University Press, New York, **1995**. (b) *Enzymes in Synthetic Organic Chemistry*; Wong, C. -H.; Whitesides, G. M., Pergamon: N. Y., **1994**. c) *Biotransformations in Preparative Organic* Chemistry; Faber, K., Ed.; Springer-Verlag: Heidelberg, 2nd. ed.,**1994**. (d) *Preparative Biotransformations*; Roberts, S.M., Ed.; Wiley: N. Y., **1993**.

2. (a) Keller, T. H.; Seufer-Wasserthal, P.; Jones, J. B. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 401. (b) Seufer-Wasserthal, P.; Martichonok, V.; Keller, T. H.; Chin, B.; Martin, R.; Jones, J. B. *Bioorganic & Medicinal Chemistry* **1994**, *2*, 35.

3. "Catalytic Asymmetric Synthesis", Ojima, I. (ed.), VCH Publishers, New York, 1993, Chaps. 1,2.

4. "The Organic Chemistry of Drug Synthesis", Lednicer, D.; Mitscher, L.A., vols. 1-4, Wiley, New York, 1990

5. (a) Gutman, A. L.; Meyer, E.; Kalerin, E.; Polyak, F.; Sterling, *J. Biotechnol. Bioeng.* **1992**, *40*, 760. (b) Pugniere, M.; San Juan, C.; Previero, A. *Tetrahedron Lett.* **1990**, *31*, 4883. (c) Gotor, V.; Garcia, M. J.; Rebelleo, F. *Tetrahedron Asymmetry* **1990**, *1*, 277. (d) Margolin, A. L.; Delinck, D. L.; Whalon, M. R. *J. Am. Chem. Soc.* **1990**. *112*, 2849. (e) Chenevert, R.; Desjardins, M.; Gagnon, R. *Chem. Lett.* **1990**, 33. (f) Ricca, J. M.; Crout, D. H. *J. Chem. Soc. Perkin Trans. 1* **1989**, 2126. (g) Kitaguchi, H.; Fitzpatrick, P. A.; Huber, J. E.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 3094. (h) Blevins, R. A.; Tulinsky, A. *J. Biol. Chem.* **1985**, *260*, 4264. (i) Roper, J. M.; Bauer, D. P. *Synthesis* **1983**, 1041.

6. (a) McPhalen, C. A.; James, M. N. G. *Biochemistry* **1988**, *27*, 6582 (b) Bode, W.; Papamokos, E.; Musil, D. *Eur. J. Biochem.* **1987**, *166*, 673.

7. (a) Frigerio, F.; Coda, A.; Pugliese, L.; Lionetti, C.; Menegatti, E.; Amiconi, G.; Schnebli, H. P.; Ascenzi, P.; Bolognesi, M *J. Mol. Biol.* **1992**, *225*, 107. (b) Tsukada, H.; Blow, D. M. M *J. Mol. Biol.* **1985**, *184*, 703. (c) Birktoft, J. J.; Blow, D. M. M *J. Mol. Biol.* **1972**, *68*, 187. (d) Blevins, R. A.; Tulinsky, A. *J. Biol. Chem.* **1985**, *260*, 4264. (e)Tulinsky, A.; Blevins, R. A. *J. Biol. Chem.* **1987**, *262*, 7737.

8. (a) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157. (b) Berger, A., Schechter, I. *Philos. Trans. R. Soc. London, Ser. B* **1970**, *257*, 249.

9. (a) De Zoote, M.C.; Kock-Van Dalen, A.C.; Van Rantwijk, F.; Sheldon, R.A. *J. Chem. Soc. Chem. Commun.*, **1993**, 1831. (b) ref. 1 (b) pp. 46-59. (c) ref. 1(c) pp. 298-305. (d) Beck, J.F.; Jones, J.B. *Tech. Chem. N.Y.*, **1976**, *10*, 187.

10. Jones, J. B.; Sneddon, D. W.; Lewis, A. J. *Biochem. Biophys. Acta* **1974**, *341*, 284.

11. Li, Z.; Patil, G. S.; Golubsky, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C. *J. Med. Chem.* **1993**, *36*, 3472-3480.

12. Ocain, T.D.; Rich, D.H. *J. Med. Chem.* **1992**, *35*, 451.

13. Lee, A. Y.; Hagihara, M.; Karmachrya, R.; Albers, M. W.; Schreiber, S. L. ; Clardy, J. *J. Am. Chem. Soc.* **1993**, *115*, 12619-12620.

14. Thompson, A. S.; Humphrey, G. R.; DeMarco, A. M.; Mathre, D. J.; Grabowski, E. J. J. *J. Org. Chem.* **1993**, *58*, 5886-5888.

15. Boyer, J. H. *J. Am. Chem. Soc.* **1951**, *73*, 5865-5866.

16. See discussion in footnote 17 in Hultin, P.G; Jones, J.B. *Bioorganic Chem.*, **1992**, *20*, 30.

17. Wallace, R.A.; Niemann, C. *Biochemistry*, **1963**, *2*, 824.

18. (a) '*Techniques of Solubilization of Drugs*' Yalkowsky, S. H. Ed.; Marcel Dekker Inc., N. Y., 1981. (b) Schellenberger, V.; Schellenberger, U.; Jakubke, H.-D. *Coll. Czech. Chem. Commun.*, **1988**, *53*, 2884. (c) Fischer, A.; Schwarz, A.; Wandrey, C Bommarius, A.S.; Knaup, G.; Drauz, K. *Biomed. Biophys. Acta.*, **1991**, *50*, 169.

19. Westermann, B.; Jones, J.B., unpublished work.

20. (a) Fredga, A.; Sjoberg, B.; Sandberg, R. *Acta Chem. Scand.* **1957**, *11*, 1609. (b) Okamoto, K.; Minami, E.; Shingu, H. *Bull. Chem. Soc. Jpn* **1968**, *41*, 1426.

21. Waley, S. G. *Biochem. J.* **1982**, *205*, 631-633.

22. Lee, T.; Jones, J.B. *J. Amer. Chem. Soc.*, **1996**, *118*, in press.

23. For subtilisin Carlsberg, entry 2SEC, 1.8-Å resolution; for a-chymotrypsin, entry 4CHA, 1.68-Å resolution.

24. (a) Bernstein, F. C.; Koetzle, T. F.; Williams, J. B.; Meyer, E. F., Jr.; Brice, M. D.; Rogers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* **1977**, *112*, 535. (b) Abola, E. E.; Bernstein, F. C.; Bryant, S. H.; Koetzle, T. F.; Weng, J. in *Crystallographic Databases -Information Content, Software Systems, Scientific Applications*; Allen, F. H.; Bergerhoff, G.; Sievers, R., Eds; Data Commision of the International Union of Crystallography: Bonn/ Cambridge/ Chester, **1987**, 107.

25. Hagler, A. T.; Osguthorpe, D. J.; Dauber- Osguthorpe, P.; Hempel, J. C. *Science* **1985**, *227*, 1309.

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